Translational Oncology
www.transonc.com

Volume 3 Number 4 August 2010 pp. 246–251 246

Comparison of the Side Populations in Pretreatment and Postrelapse Neuroblastoma Cell Lines[1]

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Abstract

Cancer stem–like cells have been identified in both primary tumors and in cell lines and seem to have a high degree of inherent resistance to traditional chemotherapeutic agents. Relapsed cancers including neuroblastoma are generally chemotherapy-resistant and carry a very poor prognosis. We investigated the side populations of three pairs of neuroblastoma cell lines derived from single patients at the time of their initial presentation and then at relapse after multimodality therapy. We found that the size of the side populations in the relapsed cell lines was significantly increased compared with its paired pretreatment cell line. In addition, these side population cells showed increased proliferation and were significantly more efficient at forming colonies in soft agar than their prerelapse pair. Gene expression analysis of the stem cell genes *NANOG* and *POU5F1* (*Oct3/4*) showed increased expression in the unsorted relapsed cell lines compared with pretreatment lines as well as in the side populations of the relapsed *versus* prerelapse cell line pairs. The increased size, proliferative ability, and colony-forming efficiency of the side populations of the postrelapse cell lines demonstrated in this study suggest that a population of stemlike cells is not being efficiently targeted by conventional therapy and implies that strategies to specifically target the stem cell fraction of neuroblastomas are needed to improve outcomes in this devastating childhood disease.

Translational Oncology (2010) 3, 246–251

Introduction

Neuroblastoma is the most common extracranial solid tumor of childhood, accounting for 15% of pediatric cancer fatalities annually. Despite advances in pediatric cancer therapy, cure rates for high-risk neuroblastoma continue to be poor, with a less than 40% long-term survival rate despite intensive treatment including chemoradiotherapy and bone marrow transplantation. On recurrence, neuroblastoma carries a high level of therapy resistance and an extremely poor prognosis with an almost universally fatal outcome [1–3].

Most cancers have been shown to contain a subpopulation of cells that exhibit stem cell–like properties. This observation has led to the formulation of the cancer stem cell hypothesis that states that tumors contain a small population of cells that have the capacity to self-renew and to differentiate, thus giving rise to the heterogeneous tumor phenotype. Although evidence suggests the existence of cancer stem cells, the hypothesis remains controversial, particularly with regard to solid tumors [4–6]. The significance of the cancer stem cell hypothesis is that reports have shown that the putative cancer stem cells have increased chemoresistance and are likely responsible for clinical relapse [7]. Thus, eradicating tumors may be difficult because conventional treatments target the bulk of the tumor cells, leaving behind the cancer stem–like cells, which, like their normal counterparts, maintain the tumor tissue. According to this hypothesis, identifying and eliminating cancer stem cells will be necessary to develop more effective cancer treatments.

The cancer stem cell hypothesis was originally proposed and has been most thoroughly studied in hematological malignancies [8,9]. However,

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[1]Funding was through the Department of Pediatrics at the Uniformed Services University of the Health Sciences in Bethesda, MD, including a fellowship training research grant to Dr Thomas Newton.
Received 6 October 2009; Revised 1 February 2010; Accepted 8 February 2010

DOI 10.1593/tlo.09301

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE
01 FEB 2010

2. REPORT TYPE

3. DATES COVERED
00-00-2010 to 00-00-2010

4. TITLE AND SUBTITLE
Comparison of the Side Populations in Pretreatment and Postrelapse Neuroblastoma Cell Lines

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Uniformed Services University of the Health Sciences,Department of Pediatrics,4301 Jones Bridge Rd,Bethesda,MD,20814-4799

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR’S ACRONYM(S)

11. SPONSOR/MONITOR’S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Cancer stem?like cells have been identified in both primary tumors and in cell lines and seem to have a high degree of inherent resistance to traditional chemotherapeutic agents. Relapsed cancers including neuroblastoma are generally chemotherapy-resistant and carry a very poor prognosis. We investigated the side populations of three pairs of neuroblastoma cell lines derived from single patients at the time of their initial presentation and then at relapse after multimodality therapy. We found that the size of the side populations in the relapsed cell lines was significantly increased compared with its paired pretreatment cell line. In addition, these side population cells showed increased proliferation and were significantly more efficient at forming colonies in soft agar than their prerelapse pair. Gene expression analysis of the stem cell genes NANOG and POU5F1 (Oct3/4) showed increased expression in the unsorted relapsed cell lines compared with pretreatment lines as well as in the side populations of the relapsed versus prerelapse cell line pairs. The increased size, proliferative ability, and colony-forming efficiency of the side populations of the postrelapse cell lines demonstrated in this study suggest that a population of stemlike cells is not being efficiently targeted by conventional therapy and implies that strategies to specifically target the stem cell fraction of neuroblastomas are needed to improve outcomes in this devastating childhood disease.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**6**	

mounting evidence supports the existence of cancer stem–like cells in solid tumors as well [10–19].

Identification of cancer stem–like cells has been performed using specific differentially expressed markers and side population (SP) analysis. In acute myelogenous leukemia (AML), $CD34^{+}$ and $CD38^{-}$ cells are highly enriched for tumor-initiating potential [8,9]. CD133, the defining member of the novel pentaspan transmembrane glycoprotein family, has proven to be useful in the identification of cancer stem cells in brain and colon cancers [13,14,16,17], and $CD44^{high}/CD24^{low}/Lin^{-}$ cells have been shown to isolate stemlike cells from human breast cancers [11]. Because selectively expressed markers have not been identified for many cancers, researchers have also used the differential ability of certain cells to exclude DNA binding dyes such as Hoechst 33342 and DyeCycle Violet to identify subpopulations enriched for cells with stemlike characteristics. Cells with the capacity to efflux these dyes were first identified in mouse bone marrow and were called SP cells because they fell to the "side" of the positively stained cells in flow cytometry analysis plots [20]. Since this original discovery, SP cells have been identified in a wide variety of normal tissues [21–24], tumors, and cell lines [7,25–32]. The mechanism regulating this efflux seems to be conferred, in part, through the expression of ATP binding cassette protein (ABC) transporters [33].

In neuroblastoma, cancer stem–like cells have been found in both primary tumor specimens and established cell lines and comprise 4% to 37% of the total population [7]. Previous studies measuring the expression of CD133 and CD117 have suggested a two-fold difference in the number of cancer stem cells in metastatic *versus* localized tumors (33% *vs* 14%) and in progressive tumors *versus* tumors in which remission was achieved (35% *vs* 18%) [34]. Additional studies have shown that many neuroblastoma cells express numerous different primitive neural markers including CD34, ABCG2, and nestin [7,35–37]. Results of SP analysis have been complex, with one report showing that 65% of primary tumors contain a stemlike SP, whereas another failed to identify a SP in neuroblastoma cells isolated from bone marrow metastases [7,15], suggesting that the significance of the SP in neuroblastoma remains unclear and warrants further evaluation.

Given the cancer stem cell hypothesis, it would seem logical that the proportion of SP cells within a particular cancer cell line would correlate with its tumorigenicity; however, to date, there have been no reports demonstrating this in cell lines. Here, we show for the first time that neuroblastoma cell lines have a stable SP and that the SP in postrelapse cell lines is both increased in size and shows increased tumor-initiating capacity as measured by colony-forming assay compared with its pretreatment pair. To the best of our knowledge, this report represents the first published data comparing the SPs of cell lines derived from pretreatment and relapsed tumors from the same patient.

Materials and Methods

Cell Lines

We used three paired neuroblastoma cell lines for *in vitro* investigations. These were a gift from the laboratory of Dr C. Patrick Reynolds from the Children's Hospital of Los Angeles. These include SMS-KCN and SMS-KCNR, SMS-KAN and SMS-KANR, and CHLA-122 and CHLA-136. Each pair of cell lines was derived from the same patient at the time of initial surgery and again at the time of relapse after chemotherapy. All cell lines were derived from original tumors and have not been otherwise modified. Cell lines were cultured in either RPMI 1640 or Iscove medium supplemented with fetal bovine serum as previously described [38]. All three pairs were used for SP percentage analysis; SMS-KCN and SMS-KCNR and CHLA-122 and CHLA-136 were used for all subsequent experiments.

Flow Cytometry—Analysis

Hoechst 33342 (Invitrogen, Carlsbad, CA) was added to the solution of cells at 5 μg per 5×10^{6} cells as previously described [39], and the SP was identified on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). The location of the SP on the flow histogram was confirmed using the ABCG2 inhibitor fumitremorgin C (Sigma, St Louis, MO) during initial analyses.

Flow Cytometry—Cell Sorting

Vybrant DyeCycle Violet stain (Invitrogen) was added to the cell suspension at 10 μM per 5×10^{6} cells as previously described [39]. After identification of the SP, the SP and non-SP cells were sorted using the BD FACS Aria Flow Cytometer (BD Biosciences). The SP cells and non-SP cells were then used for gene expression analysis as previously described and for *in vitro* growth, colony-forming, and cell proliferation assays.

In Vitro Growth Assay

Equal numbers (2.5×10^{5}) of sorted SP and non-SP cells were placed in growth medium and were allowed to proliferate under the same conditions. Samples were counted using the Beckman Coulter Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Inc, Brea, CA) every third to fifth day to estimate the cell population. After 4 weeks, cell subpopulations were counted and reanalyzed by flow cytometry.

Colony-Forming Assay

Equal numbers (2000) of sorted cells were suspended in growth medium with 3% agarose and were then plated in six-well plates. After 7 days, the number of colonies per 4× high-power field was counted in 20 random fields to determine the mean colony-forming units of each sample.

Real-time Reverse Transcription–Polymerase Chain Reaction

We measured gene expression levels of six genes: ABC transporter genes *ABCA3*, *ABCB1*, *ABCC1*, and *ABCG2* and the stem cell pluripotency transcription factors *NANOG* and *POU5F1*. 18S Ribosomal RNA was used as an endogenous control. Total RNA was extracted using the TRIzol Reagent (Molecular Research Center, Inc, Cincinnati, OH). Gene expression was measured using a one-step real-time polymerase chain reaction on the ABI 7500 Real-Time PCR System (Applied Biosystems, Inc, Carlsbad, CA). Relative differences between samples were determined using the $\Delta\Delta C_t$ method according to the manufacturer's instructions.

Statistical Analysis

Comparison between two means was performed using the Student's *t* test. $P < .05$ was considered statistically significant. All statistical analyses were performed using Prism 5.0 and/or InStat 3.1a software (GraphPad Software, Inc, La Jolla, CA).

Results

Postrelapse Neuroblastoma Cell Lines Contain a Stably Increased SP

We performed SP analysis on the three pairs of case-controlled prerelapse and postrelapse neuroblastoma cell lines. In each case, we found

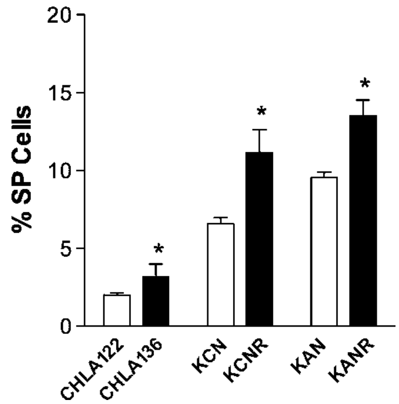

Figure 1. Percentage of SP cells from prerelapse and postrelapse cell line pairs showing a consistent increase in the relapsed cell line in every case. White bars represent the prerelapse cell lines, whereas the black bars are the postrelapse lines. Graph portrays means of multiple experiments (three or more for each cell line) performed over time. *$P < .01$ for all three pairs.

that the postrelapse cell lines maintained a significantly increased SP compared with its paired pretreatment cell line: SMS-KANR 13.5% ± 1.0% *versus* SMS-KAN 9.6% ± 0.7% ($P < .01$), SMS-KCNR 11.1% ± 1.5% *versus* SMS-KCN 6.7% ± 0.9% ($P < .01$), and CHLA-136 3.2% ± 0.8% *versus* CHLA-122 2.0% ± 0.4% ($P < .01$; Figure 1). For several months and multiple repeated experiments, the percentage of cells in the SP remained relatively stable for each cell line. This consistency was found both during analysis on the BD LSRII and during sorting on the BD FACS Aria Flow Cytometer. These results were also consistent regardless of whether cells were cultured in serum-free medium or in medium supplemented with fetal bovine serum.

SP Cells Show Increased Proliferation Rate and Colony-Forming Ability

To determine whether the non-SP and SP cells show inherent differences in growth potential, we regrew cells after sorting. We found that only the SP cells could both proliferate and recapitulate the original cell line. During a 4-week period, the non-SP cells showed minimal expansion. In contrast, during the same 4-week period, the total number of the SP cells increased in all cell lines, ranging from a doubling to a 19-fold increase (Figure 2). Not only did the SP cells proliferate but also, after several weeks, they morphologically resembled the original cell line from which they were derived. These cells were reanalyzed to determine the percentage of SP *versus* non-SP cells after 4 weeks. The non-SP cells remained without any evidence of a SP in all cases, whereas the SP cells differentiated to include both SP and non-SP populations. The SMS-KCN SP differentiated to 2% SP and 98% non-SP, whereas the SMS-KCNR SP had 11% SP and 89% non-SP, which was similar to the original cell line composition (data not shown).

We also investigated the ability of the sorted SP and non-SP cells to form colonies in soft agar. After 1 week, the sorted SP cells from the cell

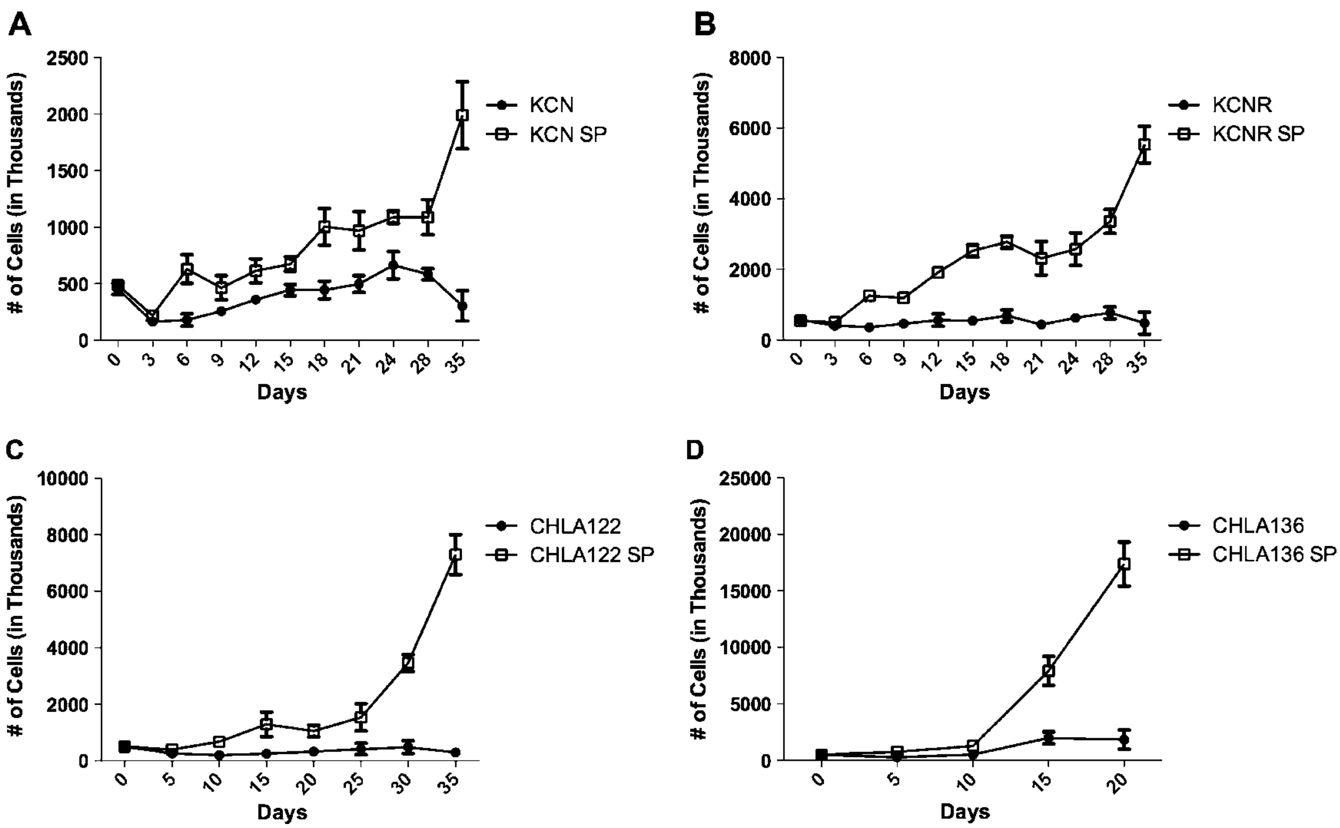

Figure 2. Graphs showing growth plots of SP cells compared with the non-SP cells after sorting. In each case, the SP cells were able to proliferate, whereas the non-SP cells did not significantly proliferate *in vitro* for up to 5 weeks after sorting. Results shown are the means of at least three experiments. (A) SMS-KCN. (B) SMS-KCNR. (C) CHLA-122. (D) CHLA-136.

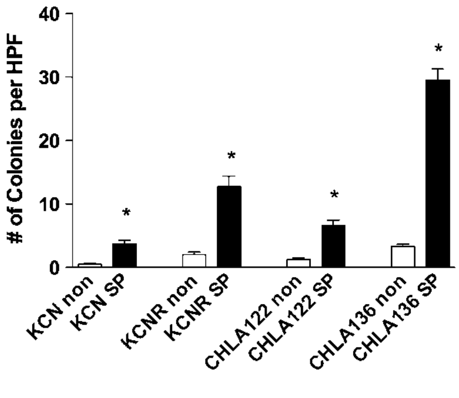

Figure 3. Results of soft agar colony-forming assays. SP cells have significantly increased colony-forming ability compared with the non-SP cells in every case. *P < .01 for all four cell lines tested. Results shown are the means of at least three experiments.

lines all showed statistically significantly (all P < .01) increased colony-forming ability compared with the non-SP cells from the same line. This ranged from a 5.4-fold to an 8.9-fold increase in colony-forming ability (Figure 3). This is consistent with the previously reported fourfold to fivefold difference between I-type (intermediate cell felt to represent a cancer-initiating cell) and N-type (neuronal type cell) neuroblastoma cells [18].

Proliferation and Colony Formation Is Increased in Postrelapse Cell Line SP Cells

The SP cells from the relapsed cell lines SMS-KCNR and CHLA-136 both showed increased proliferation rates and increased colony-forming ability compared with the SP cells from their paired prerelapse cell lines (Figure 4, *A* and *B*). The postrelapse SP cells from both postrelapse cell lines showed approximately a twofold increase in proliferation compared with the prerelapse SP cells (P < .01). The postrelapse SP cells showed a 3.1-fold (SMS-KCNR) and 4.3-fold (CHLA-136) increase in colony-forming ability compared with the SP cells from their paired prerelapse cell line (P < .01; Figure 4*C*).

Increased Expression of Stem Cell–Related Genes in Postrelapse Cell Lines

We sought to determine whether the postrelapse cell lines had an increased expression of stem cell–related genes, as might be expected given the higher proportion of SP cells found in postrelapse cell lines and the currently prevalent hypothesis that cancer stem cells may be responsible for cancer relapse. We measured the expression of two critical stem cell regulatory genes, *NANOG* and *POU5F1* (previously known as *Oct3/4*), in our three cell line pairs. In each case, we found significantly increased gene expression levels in the postrelapse line compared with its paired prerelapse line. This expression difference was 1.6- to 2.9-fold for *NANOG* and nearly 2-fold for *POU5F1* (Figure 5, *A* and *C*). We also examined the expression in the SP cells compared with that in the non-SP cells and found that these genes were consistently increased in the SP cells compared with the non-SP fraction. Postrelapse SP cells showed a 1.5- to 8-fold increased expression of both genes compared with the prerelapse SP cells from their paired cell line (Figure 5, *B* and *D*).

Increased Expression of ABCG2 Transporter Gene in SP Cells

We measured the gene expression levels of four ABC transporter genes including *ABCA3*, *ABCB1*, *ABCC1*, and *ABCG2*. As one might expect, because the presence of a SP requires expression of the *ABCG2* transporter, we found an up to 10-fold increased *ABCG2* expression in the SP cells compared with the non-SP cells. However, no other consistent patterns of ABC transporter gene expression were seen between the cell lines (data not shown).

Discussion

Relapsed neuroblastoma remains one of the greatest challenges in pediatric oncology today; despite decades of research, relapsed high-risk neuroblastoma remains almost universally fatal. The cancer stem cell hypothesis suggests that the difficulty in permanently eradicating tumors may be because conventional treatment regimens preferentially eliminate the bulk of the progeny cells while leaving the cancer stem cells intact. SP analysis has been used successfully in many different cancers to identify a subpopulation of cells that exhibit stemlike properties and that are enriched for tumor-initiating cells. This study presents data describing the SP in several neuroblastoma cell lines and suggests that these SPs are enriched for a population of cells that exhibit stemlike characteristics. Importantly, it is the first study to present and evaluate the SP properties of an *in vitro* pretreatment *versus* postrelapse model using single-patient derived paired cell lines.

Using this resource, we have shown that the size of the SP increased significantly between the pretreatment and postrelapse cell lines and that these cell lines maintain a relatively constant ratio of SP *versus*

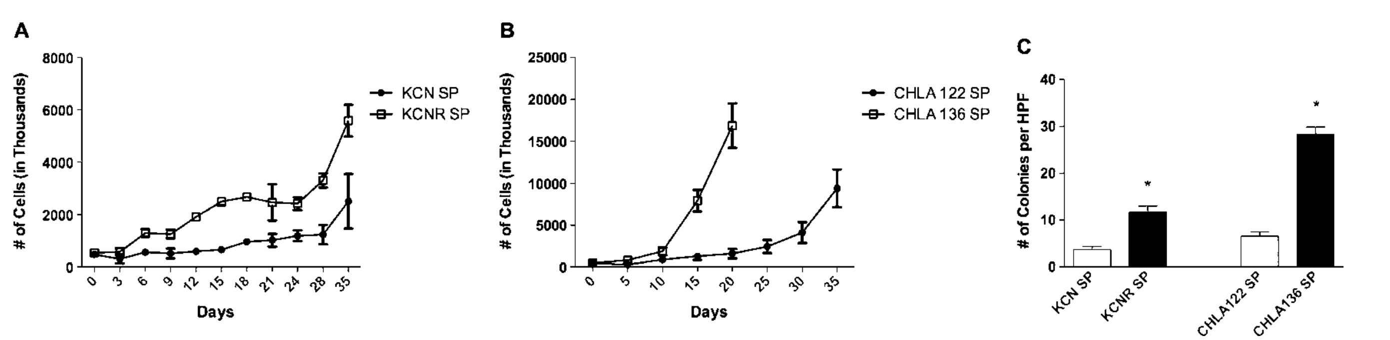

Figure 4. Comparison of growth and colony formation of prerelapse *versus* postrelapse SP cells. (A and B) Increased proliferative ability of the postrelapse SP cells from the SMS-KCNR (A) and CHLA-136 (B) cell lines compared with their paired prerelapse cell lines, respectively. (C) Postrelapse SP cells show increased colony formation efficiency compared with the prerelapse SP cells from their paired prerelapse cell lines. Results shown are means of at least three experiments. *P < .01.

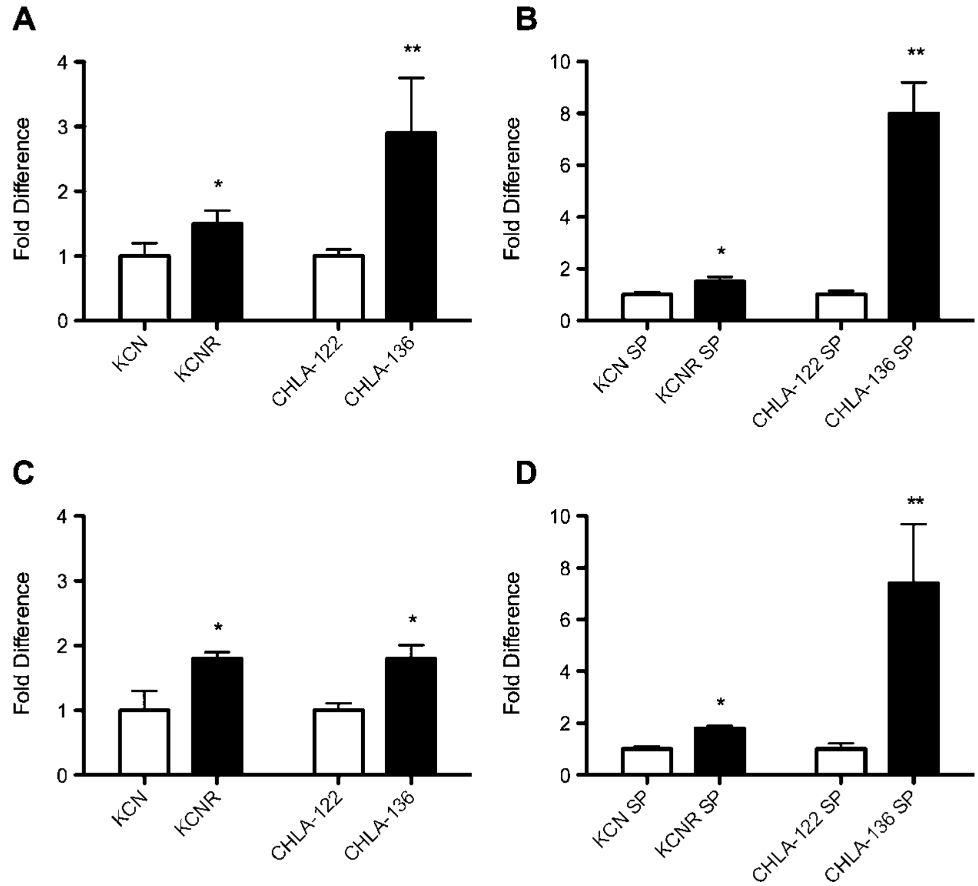

Figure 5. Expression of *NANOG* and *POU5F1* by reverse transcription–polymerase chain reaction. (A) Increased expression of *NANOG* in unsorted postrelapse cell lines compared with its prerelapse pair. (B) Increased expression of *NANOG* in the postrelapse SP cells compared with the prerelapse SP cells. (C) Increased expression of *POU5F1* in the same postrelapse cell lines compared with the prerelapse pairs. (D) Increased *POU5F1* expression in the postrelapse SP cells compared with the prerelapse SP cells. *$P < .05$, **$P < .01$.

non-SP cells over time. In addition, when reanalyzed after several weeks in culture, the SP cells had phenotypically recapitulated the original cell line in every case, whereas the non-SP cells showed little proliferative ability and had not regenerated a SP. These findings, combined with the data showing the SP cells' increased capacity to form colonies in soft agar, represent what is to our knowledge the first study to show a correlation between the percentage of SP cells within a cell line and its tumorigenicity. Studies to confirm these findings *in vivo* using xenograft models are planned.

NANOG and *POU5F1*, along with *SOX2*, have been shown to be the critical regulators of embryonic stem cell maintenance [40] and have recently been shown to be overexpressed in several different cancers [41,42]. Neither gene has been previously characterized in neuroblastoma. We found that the messenger RNA expressions of *NANOG* and *POU5F1* as well as that of the *ABCG2* transporter were significantly increased in the postrelapse cell lines compared with its paired pretreatment cell lines. Both *NANOG* and *POU5F1* also showed increased expression in the postrelapse SP cells compared with the prerelapse SP cells, suggesting that the pathways that control stem cell maintenance and expansion may play an important role in neuroblastoma relapse. Given that the messenger RNA expression of these stem cell regulators is increased in the relapsed cell lines and in the SP cell subpopulation, it seems logical that possible mechanisms for the increase in the size of the SPs include dysregulation of these pathways.

This work represents the first study to characterize the SPs in paired pretreatment and relapsed neuroblastoma cell lines. Our study supports the hypothesis that failure to eradicate the underlying stem cell population in neuroblastoma contributes to its relapse and suggests that novel agents that specifically target neuroblastoma cancer stem cells are needed if we are to improve outcomes in high-risk neuroblastoma.

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